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INTRODUCTION:

The epidermal growth factor receptor (EGFR) is a cell-surface protein that relays signals from the extracellular environment into the cell by binding specific polypeptide hormones followed by activation of intracellular signal transduction pathways. Although rarely an oncogene, the ability of EGFR-mediated signaling to generate diverse responses including growth, differentiation, stress response, apoptosis suppression, and altered mobility makes this protein a potentially powerful tumor promoter. The association between higher EGFR expression and poorer prognosis in breast cancer and the frequency of higher EGFR levels in more aggressive/metastatic breast tumors reinforce this possibility. Our research aims to uncover a link between chemotherapeutic exposure and increased expression of EGFR in breast cancer cells with the hope of explaining why higher levels of EGFR are common to more advanced breast tumors. We have shown that exposure of MCF-7, T-47D, and ZR-75-1 breast cancer cells to the anti-metabolite compound methotrexate causes an up-regulation of EGFR receptor expression. Our work demonstrates that the EGFR up-regulation usually occurs at both the mRNA level and protein level (with increased expression on the cell surface) and that this may be accompanied by changes in the expression of EGFR ligands. We also demonstrate that the methotrexate-induced EGFR expression in these cells alters EGF-mediated phosphorylation of ERK and AKT (causing changes in the specificity, timing and intensity of EGFR signaling through these pathways in a cell specific manner). ERK and AKT signaling pathways have been shown to mediate anti-apoptotic effects. We therefore hypothesize that increased EGFR expression and signaling in these cells provides a survival advantage by suppression of chemotherapy induced apoptosis, possibly explaining the prevalence of EGFR over-expression in drug resistant cell lines and more aggressive breast tumors.

Task 1: Characterize the chemotherapy-induced changes in EGFR expression in breast cancer cell lines. (months 1-12)

- Screen different cell line/compound combinations using a fluorescent reporter gene placed under the control of the EGFR promoter. (months 1-6)
- Confirm the results from the GFP-screening method using RNase protection assays to demonstrate increases in EGFR mRNA under the same conditions (months 2-6)
- Determine if the observed changes in EGFR mRNA result from altered message stability using actinomycin-D and RNase protections assays to compare EGFR mRNA half life in treated and untreated cells. (months 6-9)

- Assess requirements for *de novo* protein synthesis by combining cyclohexamide pre-treatments with RNase protection assays. (months 6-9)
- Correlate changes in protein levels with changes in EGFR mRNA levels using western blot and immunohistochemistry methods. (months 6-12)

Task 2: Determine the effects EGFR-signaling in the growth and/or survival of breast cancer cells challenged with chemotherapeutic compounds. (Months 12-24)

- Determine the effects of EGFR-signaling on growth rate and re-plating efficiency in chemotherapy-treated breast cancer cells. (months 12-18)
- Assess to role of EGFR over-expression in promoting the survival of breast cancer cells challenged with different chemotherapeutic compounds through use of MCF-7 derived stable transfectant cell lines. (months 12-18)
- Study possible connections between chemotherapy-induced EGFR signaling and apoptosis by annexin staining and Hoescht staining protocols (in combination with conditions established in prior experiments). (months 18-24)
- Assess the role of EGFR signaling in regulating expression of the EGFR gene by observing the impact of a specific EGFR-inhibitor on the chemotherapy-induced changes in EGFR expression. (months 18-24)

Figures referred to in the following section are presented in the appendix.

Progress, Task 1:

The experiments for Task 1 were significantly redesigned in order to achieve the main objective: determination of chemotherapeutic induction of EGFR expression. Three breast cancer cell lines were chosen for this project based on these criteria: MCF-7, T-47D, and ZR-75-1. A fourth cell line that does not express ER, MDA-MD-453, was added in order to address (or eliminate) a role for ER in our experimental model. The ER and EGFR status for the selected cell lines are listed in **TABLE 1**.

To measure changes in EGFR expression in the MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cell lines, it was necessary to develop a protocol with which we could reliably detect changes in EGFR content in cells that express the receptor at very low levels. Several conventional procedures were attempted with limited

success, including immunoprecipitation, Western blotting and immunohistochemistry. We finally settled on an immunofluorescence / flow cytometry protocol that yielded consistent, reproducible results. This method involved the binding of EGFR specific-antibodies to intact living cells. The levels of antibody binding, as detected through the use of a fluorescent secondary antibody, were measured by flow cytometry. Data are presented as fluorescent units per cell, with mean peak values calculated per sample population. (This protocol was substituted for the fluorescent reporter gene protocol described in Task 1).

FIGURE 1 represents the comparative level of EGFR expression in the MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cell lines as determined through the application of this method. As shown in **FIGURE 1a**, MCF-7, MDA-MB-453, and ZR-75-1 cells exhibit very low baseline EGFR expression levels. Mean peak fluorescent values for these cell lines were consistently below 10 fluorescent units. In contrast, T-47D cells, which express a modestly higher amount of EGFR, had a mean fluorescent peak value of approximately 100 (about ten-fold greater than the other cell lines). For broader sense of context, this method was applied to the EGFR over-expressing MDA-MB-231 cell line. The mean fluorescence peak value for these cells was close to 600 fluorescent units (**FIGURE 1b**).

Several chemotherapeutic options were available for study in this project. Methotrexate (MTX), a folic acid analogue that is classified as an antimetabolite, was the chemotherapeutic agent selected for use in our research. This choice was made for several reasons. First, the compound has a long history of use in the treatment of breast cancer, providing clinical context for this study. Second, MTX was toxic to the cells only after prolonged exposures (four days and longer). This allowed a window of time for studying the effects of MTX-exposure on EGFR expression. Other chemotherapeutic agents which were more acutely toxic (adriamycin, cyclophosphamide) were more limiting in this regard. Finally, MTX is stable, easily stored (at -80°C) and has no inherent fluorescence (unlike adriamycin, for example) that would interfere with flow cytometric measurements.

As shown in **FIGURE 2**, 72-hour MTX exposure (at nM to μM concentrations) resulted in an elevation of EGFR mRNA levels in three of the four cell lines tested. MCF-7 cells exhibited a 5-10 fold increase in EGFR mRNA (**FIGURE 2a**), the strongest change observed among the four cell lines. ZR-75-1 cells displayed a 2 to 3-fold rise in EGFR mRNA (**FIGURE 2d**) following the same treatment and MDA-MB-453 cells displayed a 1.5 to 2-fold rise in EGFR mRNA (**FIGURE 2b**). In these three cell lines, the MTX concentration ranges that induced changes in EGFR mRNA corresponded to those that produced a cytostatic effect in each cell line (see **FIGURE 1**). Thus, equipotent MTX doses were found to induce an increase in EGFR mRNA

among MCF-7, MDA-MB-453 and ZR-75-1 cells. In contrast, T-47D cells did not alter EGFR mRNA levels (**FIGURE 2c**) following a 72-hour MTX treatment.

FIGURE 3 depicts the changes in EGFR cell surface protein expression detected in MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cells after 72-hours MTX treatment using this method. For MCF-7, ZR-75-1 and MDA-MB-453 cells, MTX doses that induced elevation of EGFR mRNA also produced a corresponding increase in EGFR protein expression at the cell surface. For MCF-7 cells (**FIGURE 3a**) a 3-fold increase in mean peak fluorescence was observed, while for ZR-75-1 cells (**FIGURE 3c**) the fold increase in mean peak value was approximately 2-fold. [In **FIGURE 4**, representative histograms from 72-hour MTX-dose response experiments are presented for each cell line.]

At this point, our focus shifted from mechanistic aspects to clinical.translational aspects. Thus, we did not pursue the actinomycin D/ cyclohexamide experiments outlined in Task 1 during year one.

Task 2: Determine the effects EGFR-signaling in the growth and/or survival of breast cancer cells challenged with chemotherapeutic compounds.

Because we narrowed our focus to the translational aspects of this project, the objectives designated for year two were taken on in year one.

Having established that MTX-treatment of MCF-7, T-47D, and ZR-75-1 breast cancer cells leads to an increase in EGFR expression on the cell surface, we wanted to assess whether the newly expressed receptor was functional. We also wanted to determine what downstream signaling targets might be affected by receptor activation. To address both of these issues at once, we decided to measure differences in EGF-stimulated phosphorylation (activation) of two well studied downstream signaling targets: AKT and ERK. The importance of these protein kinase molecules in growth stimulation and anti-apoptotic signaling are well documented in the literature.

MCF-7, T-47D and ZR-75-1 cells were treated with MTX for 72 hours (with a single MTX concentration selected for each cell line based on the RNase protection and immunofluorescence experiments: 5.0 μ M for MCF-7 and ZR-75-1 cells, 0.5 μ M for T-47D cells). Following the 72-hour MTX-treatment, the cells were serum starved (0.1% FBS) for 24 hours and stimulated with 10 ng/ml EGF (in serum free media). Protein was collected at several time-points following the addition of EGF (1-20 minutes) from both MTX-treated cells and untreated/serum starved control cells. Protein extracts were assayed for changes in phospho-

AKT or phospho-ERK content by Western blotting, using a monoclonal antibody specific to each phosphoprotein.

As shown in **FIGURE 5**, EGF-stimulation of untreated T-47D (**FIGURE 5b**) and ZR-75-1 (**FIGURE 5c**) cells resulted in a slight increase in AKT phosphorylation over the 20-minute time-course. This indicates that the normally low levels of EGFR could be stimulated to produce a measurable effect on the ERK and AKT pathways in these cell lines. MTX treatment greatly enhanced this response in both the T-47D and ZR-75-1 cell lines. EGF-stimulated AKT activation was especially pronounced in MTX-treated ZR-75-1 cells. These observations are consistent with the elevation of EGFR expression in the MTX treated cells compared to untreated controls. In MCF-7 cells, no AKT activation was detected in either MTX-treated or untreated control cells (**FIGURE 5a**).

For ERK-phosphorylation, MCF-7, T-47D and ZR-75-1 lines exhibited detectable responses to EGF as shown in **FIGURE 6**. In untreated controls cells from each cell line, a moderate but clear increase in phospho-ERK could be detected over the 20-minute time-course. MTX treatment in all three cell lines enhanced EGF-stimulated phosphorylation of ERK (**FIGURE 6a-c**). Again, these results reflect the greater MTX-induced EGFR expression that causes a greater capacity for signaling responses through this receptor. Collectively, this data demonstrates that the EGFR produced in response to MTX exposure in MCF-7, T-47D, and ZR-75-1 cells is functional and capable of stimulating signaling pathways involving ERK and AKT.

For the remainder of these experiments, only the ZR-75-1 cell line was employed. This choice was made based upon the above data and the need to streamline the experimental workload.

To explore the effectiveness of PD153035 in our system, and to further establish the role of EGFR in the EGF-stimulated phosphorylation of ERK and AKT in MTX-treated ZR-75-1 cells, the Western blot experiments described in **FIGURES 5** and **6** were modified to incorporate a 4-hour 1 μ M PD153035 pretreatment. As shown in **FIGURE 7**, the EGF-mediated phosphorylation of AKT in ZR-75-1 cells was almost completely blocked by pretreatment with 1 μ M PD153035. This was the case for both MTX-treated and untreated control cells. A similar result is seen when phospho-ERK is assessed, as shown in **FIGURE 8**, although in the MTX-treated samples the inhibition of ERK phosphorylation in the PD153035 pretreated cells was not as complete (a slight retention of phospho-ERK is seen).

Our first experiments were designed to prove that MTX exposure does lead to apoptosis in ZR-75-1 cells and that this effect could be measured reliably using the annexin-V protocol. **FIGURE 9a** depicts the onset of apoptosis in ZR-75-1 cells treated with increasing concentrations of MTX (0.005 μ M to 50.0 μ M). Annexin-V staining is observed in cells treated with MTX concentrations that strongly affected growth (data not

shown). Given this result, we decided to narrow our focus to a single representative MTX concentration. Treatment with 5.0 μ M MTX induced apoptosis to the same degree, and with the same timing, as that seen for MTX doses one log fold higher or lower. Thus, the experiment performed for several MTX concentrations in **FIGURE 9a** was repeated for 5.0 μ M MTX three times, and the combined results presented in **FIGURE 9b**.

Having obtained both a baseline percentage value and timing for MTX-induced apoptosis in ZR-75-1 cells treated with 5.0 μ M MTX, we next wanted to determine whether this effect could be influenced by EGFR stimulation or inhibition. To address this question, ZR-75-1 cells were treated with 5.0 μ M MTX for seven days, with EGF (10 ng/ml), PD15335 (1 μ M), or both EGF and PD153035 added for the final two days of the seven-day time-course. We chose to add the EGF/PD153035 after five days MTX exposure based on the apoptosis time-course established in **FIGURE 9b**. The intent was to stimulate or inhibit EGFR just as significant levels of MTX-induced apoptosis were taking place.

As shown in **FIGURE 10** stimulation and inhibition of EGFR had differing effects on MTX-induced apoptosis in these cells. **FIGURE 10** depicts the results from a representative experiment, where the data combines six replicates (three independent repeats of this experiment were performed). **FIGURE 10a** represents percentage of viable cells after the seven-day 5.0 μ M MTX treatment. Not surprisingly, untreated control cells were greater than 90% viable. This value drops to below 50% viable in cells treated with 5.0 μ M MTX alone for seven days. **FIGURE 10b** depicts the percentage cells entering apoptosis (as measured by annexin-V binding). Untreated control cells demonstrate very little apoptosis (less than 10%); that value rises to about 50% with the seven-day 5.0 μ M MTX treatment. Stimulation of MTX-treated cells with 10 ng/ml EGF over the final two days of the 7-day time-course resulted in a partial but significant rescue from apoptosis. With EGF treatment, percent viable shifts from below 50% to above 60%; percent apoptotic shifts from 50% to approximately 40%. This result indicates that stimulation by EGF, presumably through binding and activation of EGFR, confers a survival advantage to the MTX-treated ZR-75-1 cells.

Given the effect of EGF stimulation on counteracting MTX-induced apoptosis, we anticipated that blocking EGFR activity would produce the opposite effect. We tested this assumption by treating the cells with 1 μ M PD153035 over the final two days of the seven-day MTX time-course. Contrary to our expectations, no effect on cell viability/apoptosis was seen. The concentration of PD153035 used was the same as that shown to completely abolish EGF-stimulated activation of AKT and ERK by Western blot (**FIGURES 7 and 8**), indicating that this was an effective means of blocking EGFR signaling. Nonetheless, application of PD153035 alone had no appreciable effect on the percent viable/apoptotic values. However, when EGF and PD153035

were added together, the presence of the inhibitor completely blocked the protective effect provided by EGF. These data demonstrate that in ZR-75-1 cells treated with MTX, EGFR signaling (induced by EGF) can enhance cell survival but its absence (induced by PD153035) does not necessarily decrease cell survival. (NOTE: The cytotoxic effects of PD153035 alone on ZR-75-1 cells were assessed in a preliminary experiment, prior to those described above. We found that treatment of ZR-75-1 cells with PD153035 for up to six days had no effect in promoting apoptosis (not shown) as measured by annexin-V binding).

To verify our apoptosis data, we measured cytotoxicity using a second protocol, trypan blue exclusion assay. This protocol uses a cationic dye that distinguishes viable and dead cells based on the inability of the dye to pass through intact membranes. After 1:1 dilution in trypan blue [GIBCO, Carlsbad, CA], viable and dead cells are quantified using a hemocytometer. Cells with breached membranes admit the dye and appear blue; viable cells with intact membranes exclude the dye and appear clear. The ratios of each versus the entire population are expressed as a percentage of the whole.

As shown in **FIGURE 11a**, treatment of ZR-75-1 cells with 5.0 μ M MTX induces a strong cytotoxic effect: untreated control cells are > 80% viable, a value that falls to approximately 30% after treatment with 5.0 μ M MTX for seven days. To test whether activation or inhibition of EGFR could influence this outcome, we assayed cultures treated with 5.0 μ M MTX for seven days that were also given EGF (10 ng/ml), PD153035 (1 μ M) or both in combination over the final two days of the seven-day time-course. As seen in **FIGURE 11a**, addition of EGF over the last two days of the seven-day MTX treatment resulted in the rescue of a partial but significant percentage of the cells from cytotoxicity. The viable cell percentage shifted from about 30% with MTX alone to slightly greater than 50% with the addition of EGF.

Adding 1 μ M PD153035 over the final two days of the MTX-treatment had no observable effect on the viable cell percentage when added alone. However, when EGF and PD153035 were added together, the presence of the inhibitor completely blocked the rescue-effect produced by the addition of EGF, with no increase in viable cell percentage observed. These trends parallel the data regarding the effects of EGF +/- PD153035 on MTX-induced apoptosis in ZR-75-1 cells generated by our annexin-V binding assays. The data in **FIGURE 11a** were calculated and plotted using six replicates/condition from a single experiment; the results are representative of three independent experiments.

The results of the cytotoxicity assays demonstrate that stimulating MTX-treated ZR-75-1 cells with EGF just at the time they are entering apoptosis produces a partial but significant rescue effect. To follow up on this result, we decided to repeat the experiment, this time adding the EGF +/- PD153035 to the MTX-treated culture

prior to the onset of apoptosis. In this case, the EGF +/- PD153035 was added to the cells for the last four days of the time-course, instead of the last two days. We have already demonstrated that EGFR expression rises in MTX-treated ZR-75-1 cells prior to the onset of apoptosis. With this fact in mind, we anticipated that timing the addition of EGF to precede apoptosis (rather than coincide with apoptosis) would provide a greater protective effect. As shown in **FIGURE 11b**, this was the case. The viable cell percentage in MTX-treated ZR-75-1 cultures treated with EGF for four days shifted to over 60%. The addition of PD153035 alone for four days again had no significant effect. However, as with the two-day treatment, PD153035 was able to block the effect of EGF- stimulated rescue. In this case, the inhibition was less complete, with a small EGF-rescue effect seen despite of the presence of the inhibitor. This difference may reflect the greater stability of EGF compared to PD153035. EGF present in the media may have outlasted the PD153035 to produce a muted delayed rescue effect.

KEY RESEARCH ACCOMPLISHMENTS

Our research demonstrated the following:

- Exposure of MCF-7, MDA-MB-453, T47-D and ZR-75-1 breast cancer cells to methotrexate (MTX) induces an increase in EGFR mRNA expression within 72 hours
- Under the same MTX-treatment conditions, MCF-7, MDA-MB-453, T47-D and ZR-75-1 breast cancer cells also exhibit increased EGFR expression at the cell surface as detected by immuno-fluorescence assay
- The MTX-induced EGFR expressed at the cell surface is functional as demonstrated by increased EGF-stimulated ERK and AKT pathway activation following MTX-exposure
- MTX-treated ZR-75-1 cells are protected from MTX-induced apoptosis/cell death following EGF-stimulation (indicates a cell-survival role for EGFR); this effect is blocked using an EGFR-specific inhibitor demonstrating a clear role for EGFR-signaling in cell survival under these treatment conditions.

REPORTABLE OUTCOMES:**ABSTRACTS:**

Welch, JN, Chrysogelos, SA, Clarke, R. Epidermal Growth Factor Expression In Response To Chemotherapy In Breast Cancer Cells. The Susan G. Komen Breast Cancer Foundation 4th Annual Mission Conference, Washington, DC, 2000.

Expression and Function of the Epidermal Growth Factor Receptor in Breast Cancer Cells Exposed to Chemotherapy. Proc. American Association for Cancer Research 92nd Annual Meeting, New Orleans, 2000, **Welch JN**, Chrysogelos, SA, Clarke, R.

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Hilakivi-Clarke L, Cho E, deAssis S, Olivo S, Ealley E, Bouker KB, **Welch JN**, Khan G, Clarke R, Cabanes A. Maternal and Prepubertal Diet, Mammary Development and Breast Cancer Risk. *Journal of Nutrition* 131: 154S-157S, 2001.

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Gu Z, Lee RY, Skaar TC, Bouker KB, **Welch JN**, Lu J, Liu A, Davis N, Leonessa F, Brunner N, Wang Y, Clarke R. Molecular Profiles of Antiestrogen Resistance Implicate NFkB, cAMP Response Element Binding, Nucleophosmin and Interferon Regulatory Factor-1. (submitted)

CONCLUSIONS:

Our findings suggest that exposing breast cancer cells to cytotoxic drugs leads to an increase in EGFR levels, which renders the cells more responsive to EGF-stimulation and promotes cell survival through the suppression of apoptosis.

Our data demonstrates a connection between MTX-treatment and both greater EGFR-mediated signaling through ERK/AKT pathways, as well as an anti-apoptotic/survival effect. The role of EGFR signaling in ERK/AKT activation has long been established. However, our results connecting EGFR signaling with decreased apoptosis adds new data to an area of study that has only recently emerged. Whether the increased ERK/AKT signaling is directly connected to the EGF-mediated anti-apoptotic effects remains to be determined.

Based on the data presented in a number of recently published studies (see references), the association between EGFR expression and apoptosis suppression is becoming clear EGFR signaling can affect the expression and post-translational modification of pro- and anti-apoptotic proteins to influence apoptosis.

EGFR-mediated activation of AKT can suppress apoptosis through the interaction of AKT with different apoptotic signaling proteins. EGFR-mediated activation of ERK can also result in the phosphorylation and down-regulation of Bad in a manner similar to that produced by AKT. Our data demonstrate both increased capacities for EGFR-mediated ERK and AKT signaling, and anti-apoptotic effects of EGFR stimulation in MTX-treated cell lines. These results, viewed in the context of the recently published research described above, suggest a connection between ERK/AKT signaling and anti-apoptotic EGFR-mediated effects. However, more experiments will have to be performed to confirm this relationship.

Although prior research has shown that exposure of breast cancer cells to chemotherapy can result in increased EGFR and that EGFR can suppress apoptosis in a number of cancer cell lines, there is very little published research directly relating these events. In other words, no one has yet published data showing chemotherapy induced EGFR expression as a direct precursor to EGFR-mediated suppression of apoptosis. Our research appears to be unique in demonstrating that connection. In addition, we show that subtle changes in the level EGFR expression can have measurable effects on cell survival. This indicates that EGFR-targeted interventions may have a wider use in the treatment of breast cancer, including the treatment of tumors that express low but functional levels of the receptor.

- **Future Aim 1: Expansion of the Apoptosis Studies.** Although we have shown that EGF-stimulation can reverse apoptosis induced by MTX-exposure and that EGFR signaling can activate AKT and ERK under these conditions, our research has not directly addressed the specific roles of

ERK and AKT signaling in EGFR-mediated apoptosis response. This could be achieved through the use of specific inhibitors that target each pathway. In addition, possible EGFR-mediated changes in the expression of Bcl-2 family members could be investigated. If found, the role of ERK/AKT signaling in affecting the expression of pro-apoptotic and anti-apoptotic proteins could also be studied, thus clarifying the possible relationship (or lack thereof) between EGFR-mediated ERK/AKT signaling and apoptosis suppression.

- **Future Aim 2: Assess changes in susceptibility to EGFR-target therapeutics.** From the physician's perspective, determining whether the chemotherapy-induced changes in EGFR expression will render a tumor more vulnerable to EGFR-directed therapeutics may be the most important issue. EGFR-directed therapeutics have been designed to utilize a number of strategies to target cells expressing the receptor. Our data show that stimulation of EGFR following MTX-treatment reduces apoptosis. Demonstrating the reverse – that blocking EGFR will increase apoptosis – will be of far greater therapeutic value. A distinction between the effects of blocking receptor function versus targeting receptor expression with EGFR-directed toxic drugs could also be made: if blocking EGFR activity does not push the cells into apoptosis, using EGFR as a surface target for the delivery of cytotoxic agents may prove effective. The apoptosis/cytotoxicity experiments involving the EGFR-inhibitor PD153035 described in our results could be expanded to include EGFR directed therapeutics to address this aim. One final important consideration is whether the MTX-mediated induction of EGFR expression is confined to transformed/cancer cells or a response common to both normal and cancer cells. The effectiveness and specificity of using an EGFR-targeted therapy following treatment with a conventional chemotherapy such as methotrexate would be far greater if EGFR-upregulation were confined to cancer cells. Thus, the answer to this question may ultimately determine the therapeutic utility of combining conventional cancer treatments with EGFR-specific therapies.
- **Future Aim 3: Expand to include additional cytotoxic drugs.** Demonstrating whether the results generated by our research are either methotrexate-specific, or are indicative of a more general response to cytotoxic drugs, will be an important determinant of how clinically applicable combined therapeutic regimens could be. With experimental conditions largely worked out in the MTX-experiments, chemotherapeutic compounds with distinct mechanisms of action could be incorporated

into this research, including several of those tested in preliminary experiments. Selection of key experiments to undertake with each new compound, as opposed to repeating every experiment with each, would be critical to addressing this issue efficiently.

- **Aim 5: Address the mechanisms behind EGFR expression.** One issue that has remained largely unexplored is the mechanism(s) driving the increased expression of EGFR in response to MTX-treatment. MTX-treatment is known to influence the expression of the enzyme that it inhibits (dihydrofolate reductase - DHFR) and the membrane-bound protein that brings MTX into the cell (reduce folate carrier - RFC). Focusing on transcriptional elements common to promoters of these genes and that of the EGFR gene might produce promising candidates. The transcription factors p53 and Sp1, which have been reported to promote the expression of DHFR, RFC and EGFR, are two such examples. Exploring changes in EGFR promoter activity would not only clarify the question of selection versus increased expression, but might also provide more general information about the changes in gene expression that occur in response to the cellular stresses induced by cytotoxic drugs.

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APPENDIX: (Tables and Figures)**TABLE 1:** Essential characteristics of the breast cancer cell lines selected for this research.

| CELL LINE | ORGANISM | TISSUE | MORPHOLOGY |
|------------|----------|--|------------|
| MCF-7 | human | adenocarcinoma; mammary gland; breast; pleural effusion | epithelial |
| MDA-MB-231 | human | adenocarcinoma; mammary gland; breast; pleural effusion | epithelial |
| MDA-MB-453 | human | mammary gland; breast; pleural/pericardial effusion | epithelial |
| T-47D | human | ductal carcinoma; mammary gland; breast; pleural effusion | epithelial |
| ZR-75-1 | human | ductal carcinoma; mammary gland; breast; ascites; epithelial; metastatic site: ascites | epithelial |

Information collected from the 2001 American Type Culture Collection (ATCC) online catalog. (<http://phage.atcc.org>)

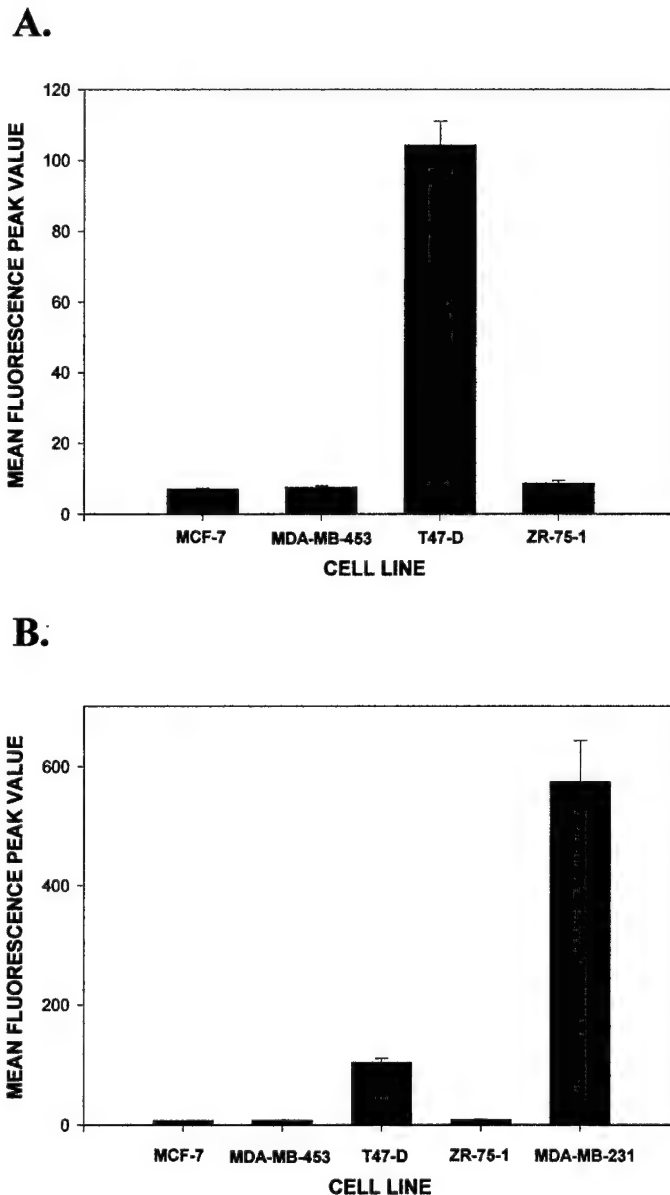


FIGURE 1: Comparison of normal-EGFR expression levels in MCF-7, MDA-MB-453, T-47D and ZR-75-1 breast cancer cell lines by immunofluorescence / flow cytometry. MCF-7, MDA-MB-435, T-47D and ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. The cells were trypsinized, rinsed twice in PBS and pelleted (1000 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence / flow cytometry. PRIMARY ANTIBODY: MAb 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell was assessed by flow cytometry and data for each cell line is presented as mean fluorescent peak values per cell population. (a) Baseline mean fluorescence peak values for untreated MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells; (b) the same data presented in part (a) with the EGFR over-expressing MDA-MB-231 cell line added for comparison.

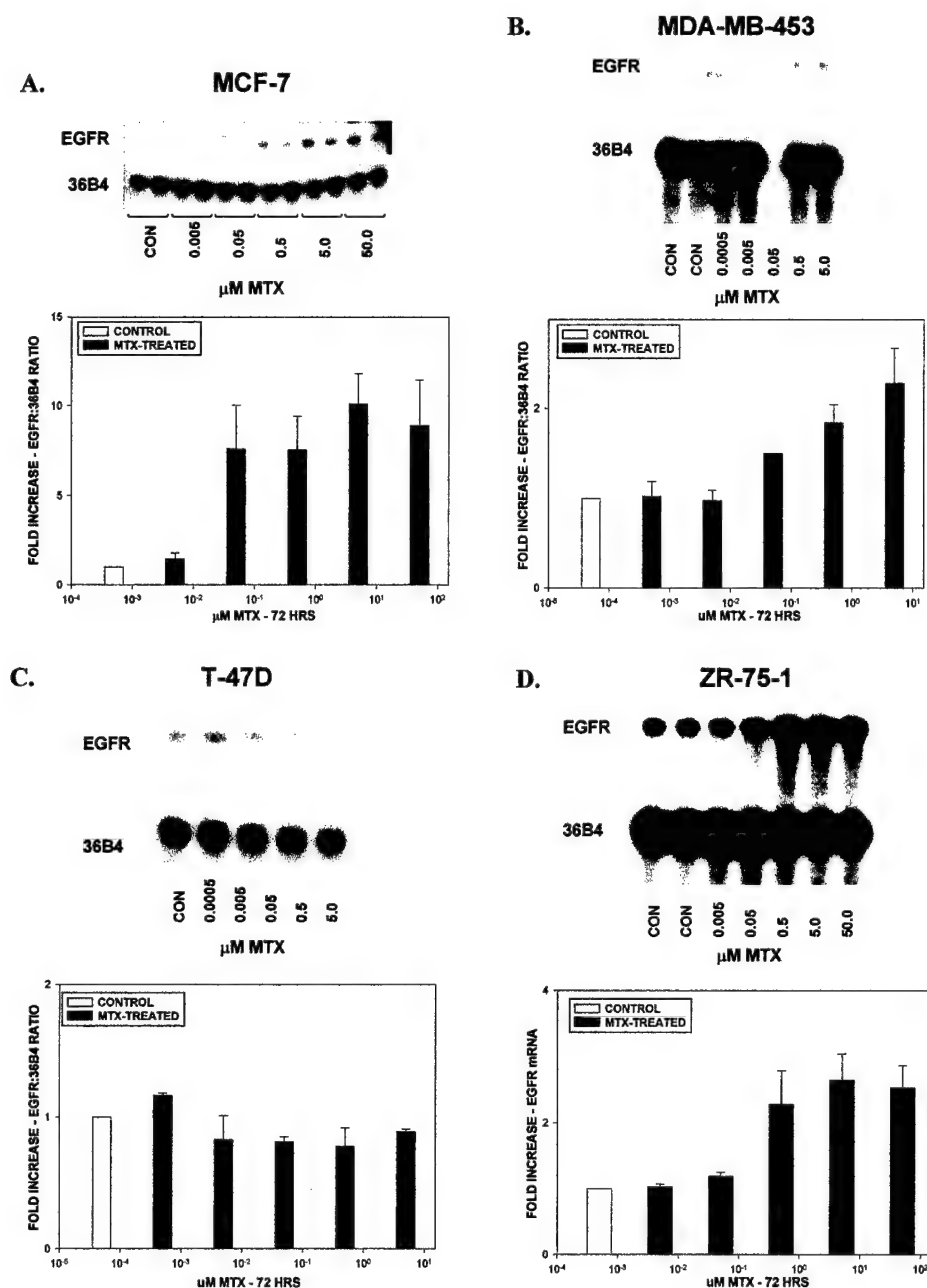


FIGURE 2: Comparison of normal-EGFR expression levels in MCF-7, MDA-MB-453, T-47D and ZR-75-1 breast cancer cell lines by immunofluorescence / flow cytometry. MCF-7, MDA-MB-453, T-47D and ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. The cells were trypsinized, rinsed twice in PBS and pelleted (1000 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence / flow cytometry. PRIMARY ANTIBODY: MAb 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell was assessed by flow cytometry and data for each cell line is presented as mean fluorescent peak values per cell population. (a) Baseline mean fluorescence peak values for untreated MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells; (b) the same data presented in part (a) with the EGFR over-expressing MDA-MB-231 cell line added for comparison.

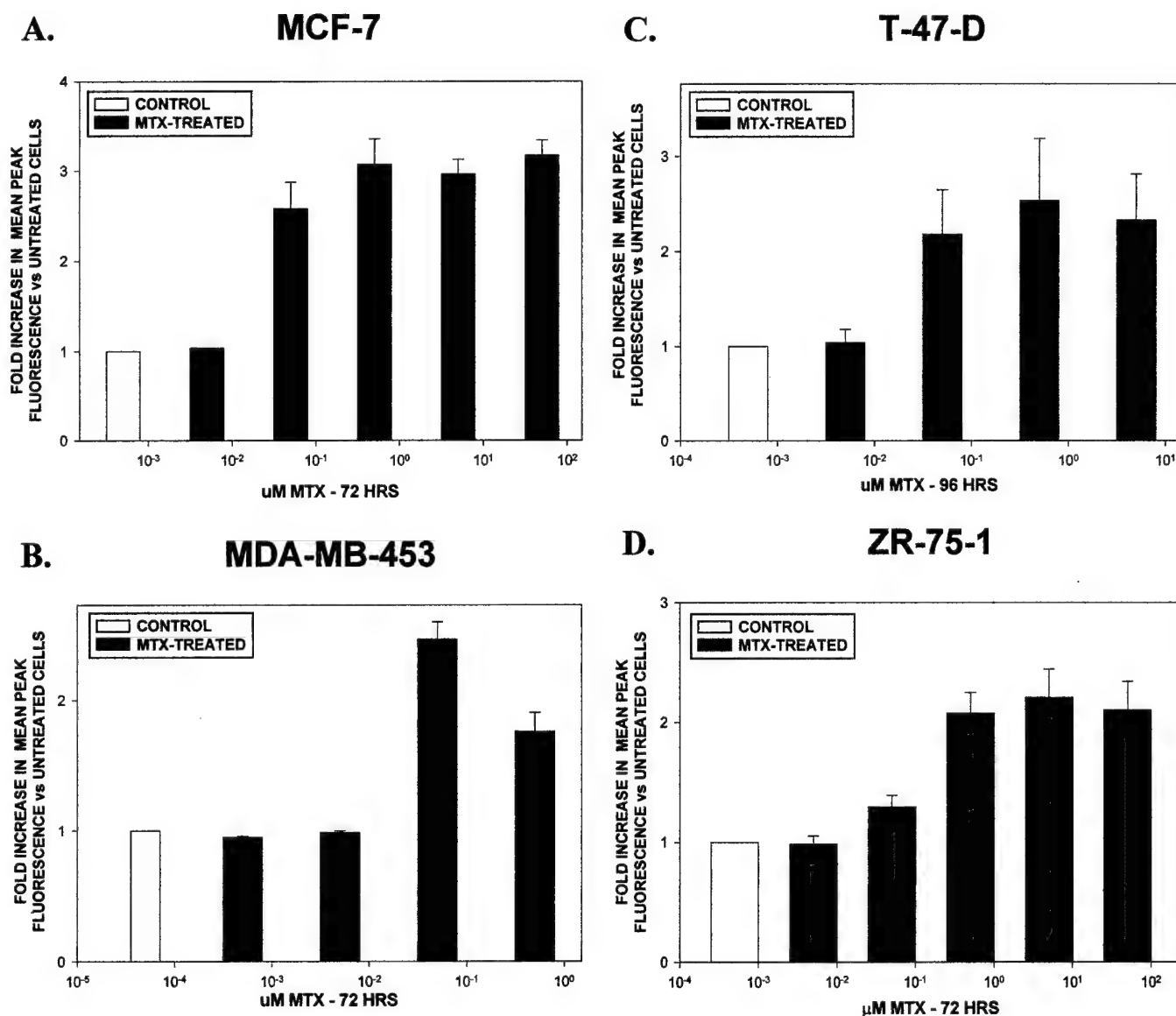


FIGURE 3: MTX-induced changes in EGFR surface expression in MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells. (a) MCF-7, (b) MDA-MB-453, (c) T-47D, and (d) ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. Methotrexate (MTX) was added to the cultures (concentration range 0.005 to 50.0 μ M) and the cells were grown for 72 hours. The cells were trypsinized, rinsed twice in PBS and pelleted (100 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence. PRIMARY ANTIBODY: Mab 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell assessed by flow cytometry. Data is presented as fold increases in mean peak fluorescence per sample for each MTX treatment (blue) vs. untreated control cells (white). Data from five separate experiments are combined. Error bars represent mean \pm standard error; $n \geq 3$.

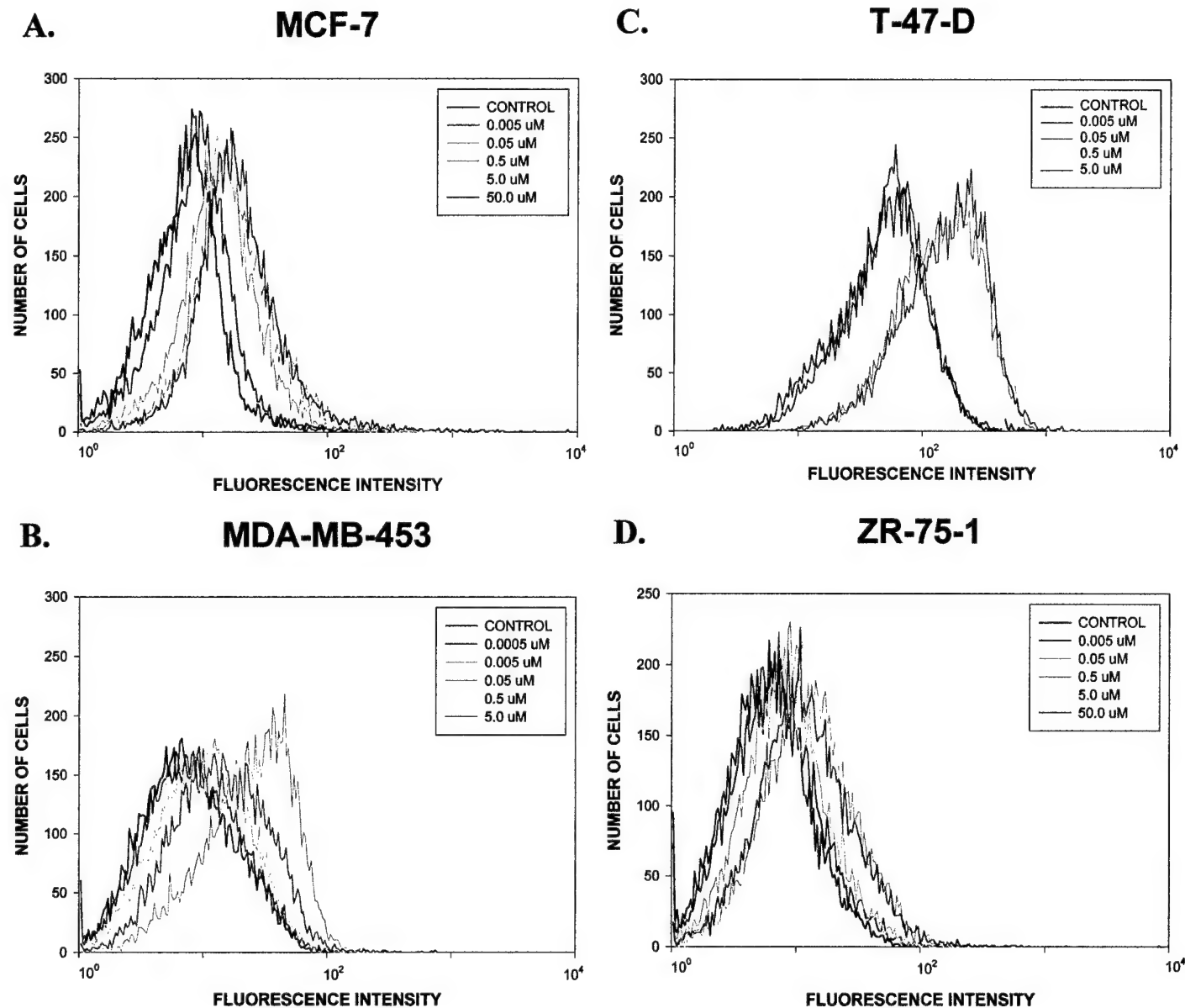


FIGURE 4: Representative histograms for the immunofluorescence / flow cytometry data regarding MTX-changes in EGFR surface expression. Representative histograms generated by the immunohistochemistry / flow cytometry protocol used to detect EGFR surface expression in (a) MCF-7, (b) MDA-MB-453, (c) T-47D and (d) ZR-75-1 cells. Details about this experiment method are presented in the legend for **FIGURE 3**.

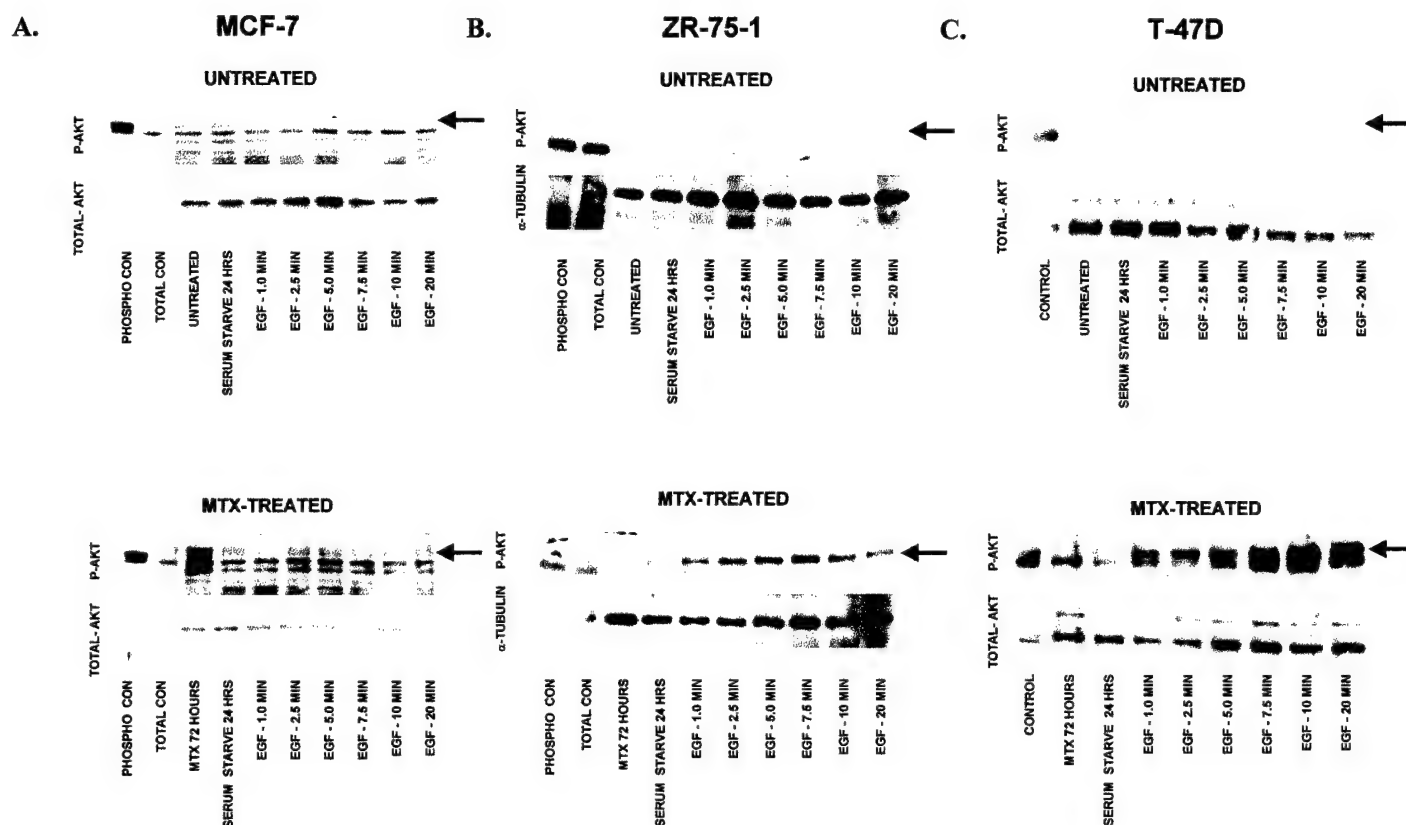


FIGURE 5: EGF-stimulated activation of AKT in MCF-7, T-47D and ZR-75-1 cells with and without MTX-treatment. (a) MCF-7, (b) ZR-75-1, and (c) T-47D breast cancer cells were plated in T-75 flasks and grown to approximately 60% confluence. The cells were treated with 0.5 or 5.0 μ M MTX for 72 hours in 10% FBS/IMEM, rinsed twice with 1X PBS, serum starved (in 0.1% FBS/IMEM) for 24 hours, and then treated with 10 ng/ml EGF for 1-20 minutes. At each time-point, the cells were rinsed twice with 1X PBS and harvested in cell lysis buffer (Cell Signaling) with 1 mM PMSF added immediately before use. Samples were stored at -80°C and assayed for protein concentration by BCA protocol. 10-20 μ g/sample were electrophoresed on 8% tris-glycine gels (120V) and blotted onto immobulin-P membranes. Primary antibody: Phospho-AKT 1:1000/PBST (Cell Signaling); Secondary Antibody: anti-rabbit 1:5000/PBST (Cell Signaling); ECL detection (Amersham). The blots were stripped and re-probed for total AKT or α -tubulin (depending on the cell line) to serve as loading controls. Each blot shown is representative of at least three individually repeated experiments. (Arrows indicate the expected location of the phospho-specific AKT band. The presence of this band the lanes for our purchased controls was somewhat variable owing, we expect, to instability of the phospho-protein after multiple freeze-thaw cycles. However, the presence of the lower band was consistent in the control lanes and served adequately as a size marker for these experiments.)

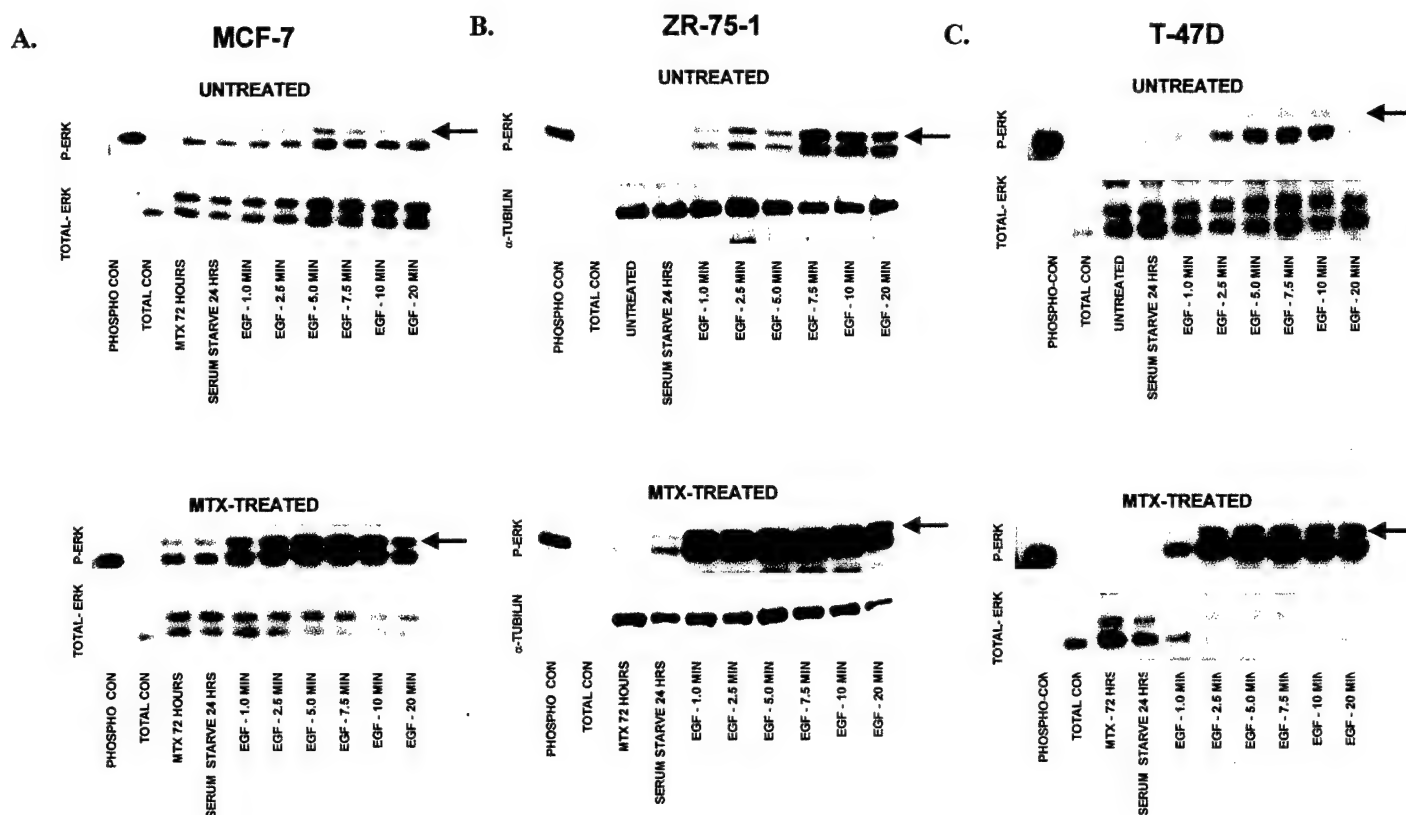
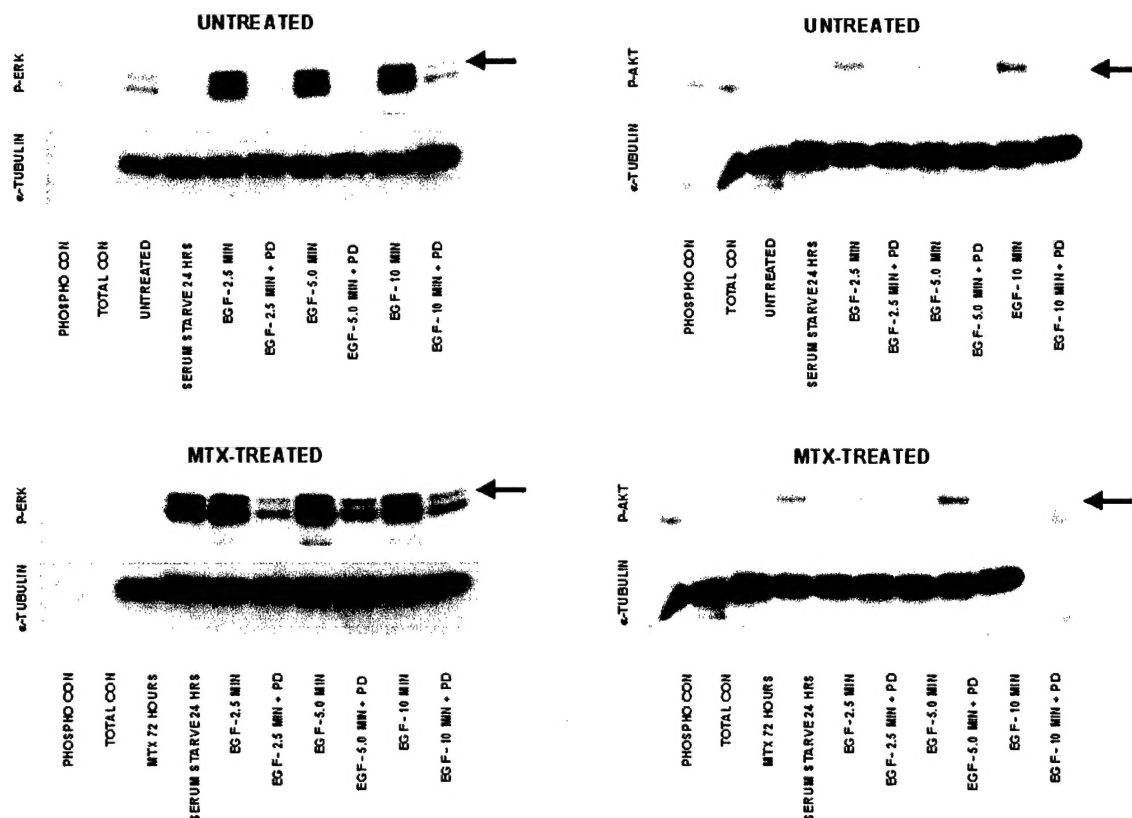


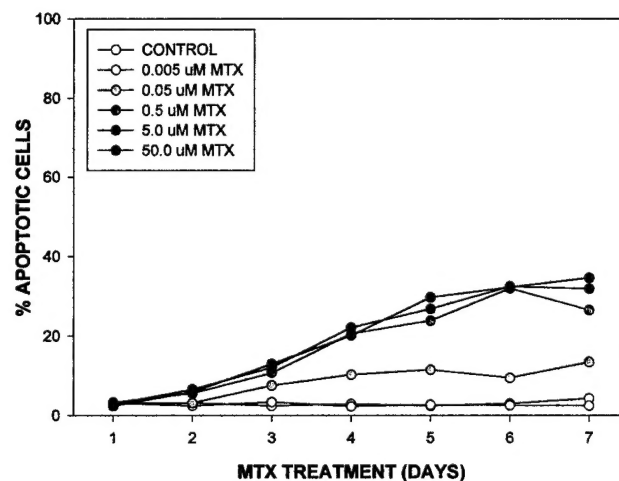
FIGURE 6: EGF-stimulated activation of ERK in MCF-7, T-47D and ZR-75-1 cells with and without MTX-treatment. (a) MCF-7, (b) ZR-75-1, and (c) T-47D breast cancer cells were plated in T-75 flasks and grown to approximately 60% confluence. The cells were treated with 0.5 or 5.0 μ M MTX for 72 hours in 10% FBS/IMEM, rinsed twice with 1X PBS, serum starved (in 0.1% FBS/IMEM) for 24 hours, and then treated with 10 ng/ml EGF for 1-20 minutes. At each time-point, the cells were rinsed twice with 1X PBS and harvested in cell lysis buffer (Cell Signaling) with 1 mM PMSF added immediately before use. Samples were stored at -80°C and assayed for protein concentration by BCA protocol. 10-20 μ g/sample were electrophoresed on 8% tris-glycine gels (120V) and blotted onto immobulin-P membranes. Primary antibody: Phospho-ERK 1:1000/PBST (Cell Signaling); Secondary Antibody: anti-rabbit 1:5000/PBST (Cell Signaling); ECL detection (Amersham). The blots were stripped and re-probed for total ERK or α -tubulin (depending on the cell line) to serve as loading controls. Each blot shown is representative of at least three individually repeated experiments.



LEFT: FIGURE 7: Inhibition of EGF-stimulated activation of AKT in MTX-treated ZR-75-1 cells by PD153035. ZR-75-1 breast cancer cells were plated in T-75 flasks and grown to approximately 60% confluence. The cells were treated with 5.0 μ M MTX for 72 hours in 10% FBS/IMEM, rinsed twice with 1X PBS, serum starved (in 0.1% FBS/IMEM) for 24 hours. Flasks were divided into two groups (with controls set aside). One set was treated with 1 μ M PD153035 for four hours prior to the addition of EGF. The other was not given PD153035. The cells were rinsed to remove the PD153035 after 4 hours and treated with 10 ng/ml EGF for 2.5 - 10 minutes. At each time-point, the cells were rinsed twice with 1X PBS and harvested in cell lysis buffer (Cell Signaling) with 1 mM PMSF added immediately before use. Samples were stored at -80°C and assayed for protein concentration by BCA protocol. 10-20 μ g/sample were electrophoresed on 8% tris-glycine gels (120V) and blotted onto immobulin-P membranes. Primary antibody: Phospho-AKT 1:1000/PBST (Cell Signaling); Secondary Antibody: anti-rabbit 1:5000/PBST (Cell Signaling); ECL detection (Amersham). The blots were stripped and re-probed for α -tubulin to serve as loading control. The blot shown is representative of two separate experiments.

RIGHT: FIGURE 8: Inhibition of EGF-stimulated activation of ERK in MTX-treated ZR-75-1 cells by PD153035. ZR-75-1 breast cancer cells were plated in T-75 flasks and grown to approximately 60% confluence. The cells were treated with 5.0 μ M MTX for 72 hours in 10% FBS/IMEM, rinsed twice with 1X PBS, serum starved (in 0.1% FBS/IMEM) for 24 hours. Flasks were divided into two groups (with controls set aside). One set was treated with 1 μ M PD153035 for four hours prior to the addition of EGF. The other was not given PD153035. The cells were rinsed to remove the PD153035 after 4 hours and treated with 10 ng/ml EGF for 2.5 - 10 minutes. At each time-point, the cells were rinsed twice with 1X PBS and harvested in cell lysis buffer (Cell Signaling) with 1 mM PMSF added immediately before use. Samples were stored at -80°C and assayed for protein concentration by BCA protocol. 10-20 μ g/sample were electrophoresed on 8% tris-glycine gels (120V) and blotted onto immobulin-P membranes. Primary antibody: Phospho-ERK 1:1000/PBST (Cell Signaling); Secondary Antibody: anti-rabbit 1:5000/PBST (Cell Signaling); ECL detection (Amersham). The blots were stripped and re-probed for α -tubulin to serve as loading control. The blot shown is representative of two separate experiments.

A.



B.

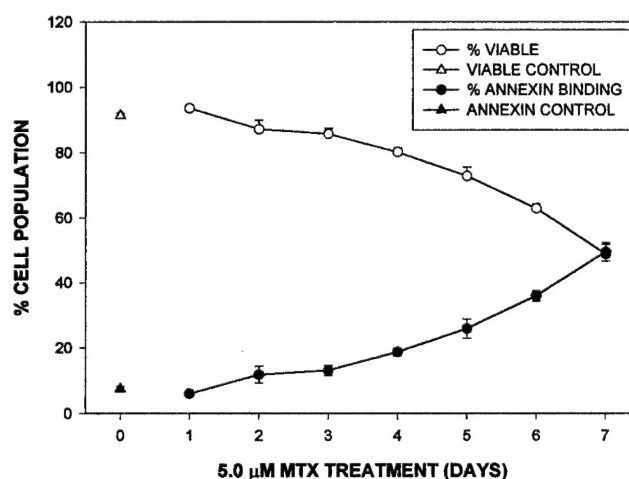


FIGURE 9: Timing of MTX-induced apoptosis in ZR-75-1 cells as measured by annexin-V staining. ZR-75-1 breast cancer cells were seeded in 6 well plates (the starting number of cells graded to allow shorter MTX-exposure doses room to grow without attaining confluence) and plated overnight. MTX (dose range 0.005 μ M to 50.0 μ M) was added to one sample on each successive day such that the samples exposed to MTX for 7 days were drugged on day one and those exposed for one day were drugged on day six. All samples were collected on day seven by trypsinization, rinsed with 1X PBS and counted. 200,000 cells were aliquoted and stained with Annexin-V to measure apoptosis. Untreated cells were used as controls. The data generated is expressed as the apoptotic percentage of the entire cell population. (a) Data from a single experiment testing the entire 0.005 μ M to 50.0 μ M MTX concentration range. (b) Combined data from three repeated experiments for 5.0 μ M MTX. This concentration was chosen for the apoptosis/cytotoxicity experiments described below. Error bars represent mean \pm standard error; $n \geq 3$.

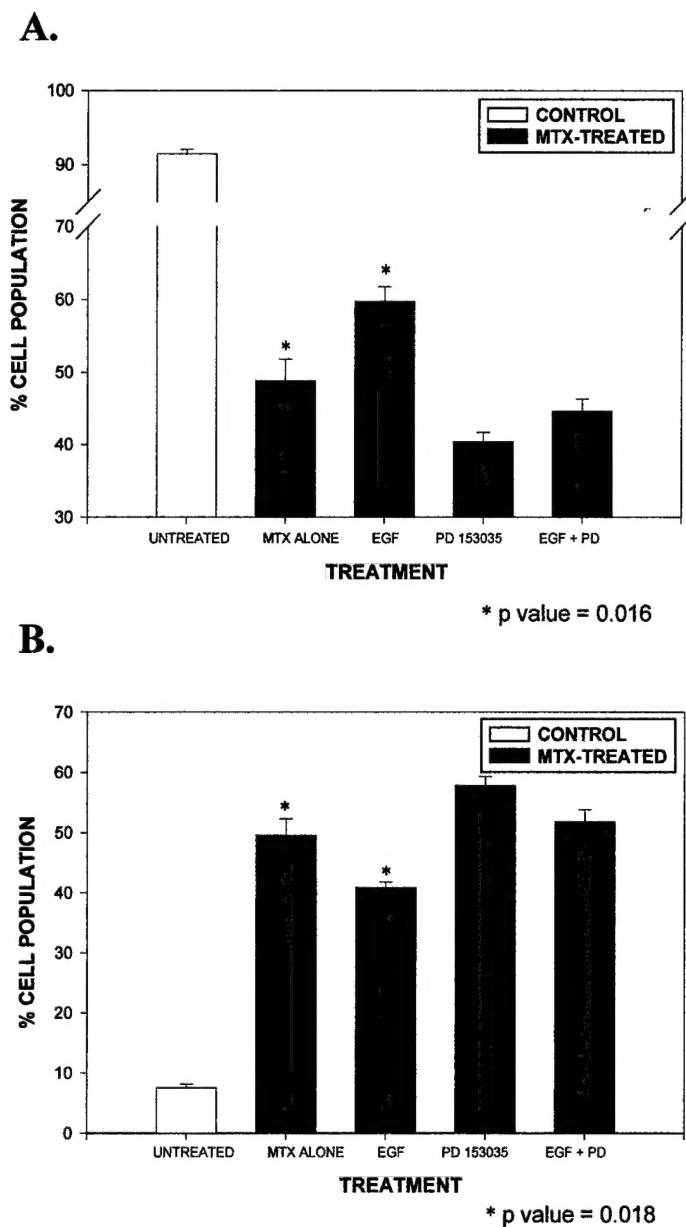
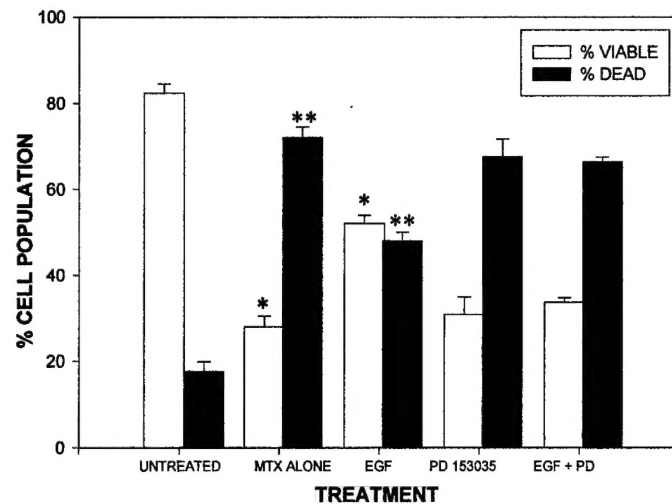
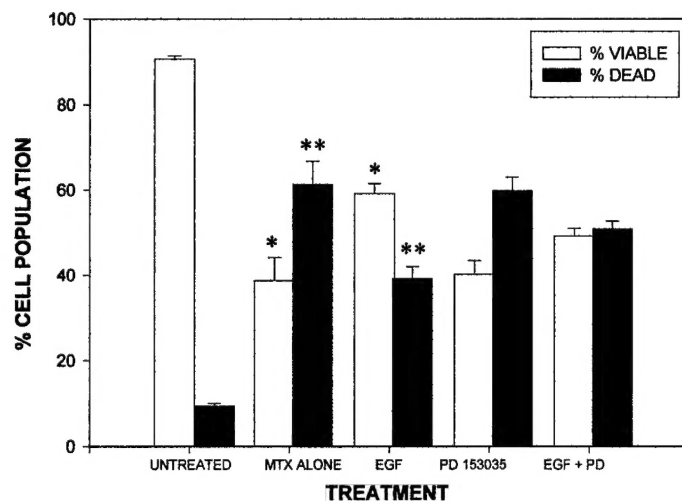


FIGURE 10: EGF-stimulation partially rescues ZR-75-1 cells from MTX-induced apoptosis as measured by annexin-V staining. ZR-75-1 cells were seeded in 6 well plates (200,000 per well; 50,000 per well for controls) and allowed to plate down overnight. 5.0 μ M MTX was added and the cells were allowed to incubate for 5 days. EGF (1 ng/ml) with or without PD153035 (1 μ M) was added to the plates without removal of the MTX-containing media, and the cells were allowed to incubate for 2 more days. Upon completion of the 7 day time-course, the cells were collected by trypsinization (adherent and floating cells). 200,000 cells per replicate were assayed for annexin-V binding (TREVIGEN ANNEXIN-V KIT). % viable cells (**a**) and % annexin-binding/apoptotic cells (**b**) are plotted. The data shown is representative of three independent experiments. Error bars represent mean \pm standard error; $n \geq 3$.

A.

* p value < 0.001

** p value < 0.001

B.

* p value = 0.006

** p value = 0.004

FIGURE 11: EGF-stimulation partially rescues ZR-75-1 cells from MTX-induced cytotoxicity as measured by trypan blue exclusion assay. (a) ZR-75-1 cells were seeded in 6 well plates (200,000 per well; 50,000 per well for controls) and allowed to plate down overnight. 5.0 μ M MTX was added and the cells were allowed to incubate for five days. EGF (1 ng/ml) with or without PD153035 (1 μ M) was added to the plates without removal of the MTX-containing media, and the cells were allowed to incubate for two more days. Upon completion of the seven-day time-course, the cells were collected by trypsinization (adherent and floating cells). Aliquots for each sample (six replicates per treatment) were diluted 1:1 in pre-made trypan blue dye. Clear (viable) and blue (membrane-compromised) cells were tallied. Percentages for each vs. the total number of cells per replicate were calculated and plotted as % viable (white) and % dead (blue). Results are representative of three independent experiments. (b) The experiment described above was repeated with the addition of EGF +/- PD153035 after three days MTX-treatment (instead of five). The doubled the length of time during which the MTX-treated cells were exposed to EGF +/- PD153035. Error bars represent mean \pm standard error; $n \geq 3$.